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ANIMAL EXPERIMENTATION

Correlation of somatic cell steroid secretion and quality of generated oocytes after in-vitro stimulation of mouse follicles*

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Abstract *Purpose:* To test the possibility of follicular somatic cell steroidogenesis as a marker for quality of their embraced oocytes.

Methods: Mechanically isolated mouse preantral follicles were cultured and matured in-vitro (IVC/IVM) for study.

Results: During IVC/IVM, oogenesis occurred concomitantly with folliculogenesis in a coordinated manner and simultaneously with progressive increments of somatic cell steroidogenesis. Follicular E_2 production of matured oocytes were significantly higher than that of immature ones. The majority of MII oocytes (32/36) and all developed blastocysts(12/12) were associated with active E_2 production prior to ovulation. In this study, 18 MII oocytes met both requirements for active and optimal E_2 production. 13 of them were fertilized and 10 developed into blastocysts.

Conclusion: Active somatic cell steroidogenesis prior to ovulation and an optimal steroid milieu at ovulation are prerequisites for generation of competent oocytes after follicular maturation in-vitro.

Keywords Follicular in-vitro maturation · Oocyte competency · Somatic cell steroidogenesis

* Predictability of somatic cell steroidogenesis for oocyte competency after follicular IVC/IVM.

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Introduction

The development of in-vitro culture/in-vitro maturation (IVC/IVM) of follicles to generate mature oocytes holds much attraction for clinical practice, animal production and research. In-vitro systems that utilize preantral follicles as a source for oocytes have been developed for many species such as mice [1–10), cat [11], cow [12, 13], pig [14], rodents [15], porcine [16] and even humans [17]. Though in-vitro maturation of follicles have been successful in developing viable blastocysts [1–4] and even in producing live young [14, 18], production of in-vitro blastocysts and offspring still remains relatively low. Besides, live-births resulting from in-vitro maturation of follicles were only reported in mouse [18] and pig [14]. Further, the methods that have proven to be successful for the culture of isolated mouse follicles are not able to support the growth of large human follicles at all. Therefore, markers for improving the efficiency of mouse follicle maturation, and strategies for perfecting in-vitro maturation of human follicles are in need. It is our hope to develop an optimal IVC/IVM system which can be used as an in-vitro model for studying the mechanism of folliculogenesis, oogenesis, embryogenesis, and the interaction between follicular cells and oocytes.

A simple culture system for in-vitro maturation of early preantral mouse ovarian follicles has been established and used in our Lab [1, 2]. After mechanical isolation, follicles were cultured singly in microdroplets under oil in medium supplemented with recombinant FSH and LH at 37°C and 5% CO_2. During in-vitro maturation, cultured follicles underwent dramatic morphological changes, which ultimately led to the formation of antral follicles and the production of oocytes. As follicles grew and matured progressively, they also gradually increased their daily E_2 secretion [1, 2]. However, even under the same culture conditions, these cultured

follicles did not proceed homogeneously and usually generated oocytes with heterogenous qualities, ranging from degenerated, immature (at the GV stage), and matured (i.e., GVBD or MII) oocytes. Meanwhile, these follicles exhibited great variation in their daily E_2 secretions with different secretion patterns.

During folliculogenesis, follicular cell support is essential to provide germ cells with nutrients and growth regulators to ensure the progression of oocyte maturation. Conversely, oocytes actively promote growth and differentiation of follicular cells [15]. Well synchronization between somatic cell differentiation and oocyte maturation will then induce proper nuclear and cytoplasmic maturation in the fully grown oocytes to ensure its competency [15]. Therefore, at any stage, if somatic cells decrease or cease their function of secreting hormones and growth factors, or if oocytes resist or stop their response to the somatic cells, the process of follicular maturation will be compromised and the competence of the oocytes generated will be greatly affected. Such a close relationship between somatic cell physiological function and oocyte maturation led us to wonder whether somatic cell steroidogenesis can be an indicator for the competency of their embraced oocyte.

Our IVC/IVM system allows us to mature individual follicles in microdroplets, to monitor follicular growth and to assess oocyte quality under the microscope and to have an accurate measurement of steroid production from individual follicles. Therefore, this system would be a perfect model to study the correlation between individual follicular steroidogenesis and the quality of the corresponding oocytes.

The purpose of this study was to confirm the role of somatic cell steroidogenesis during folliculogenesis, to find an optimal steroid milieu for quality oocyte generation and to assess the predictability of somatic cell steroidogenesis for blastocyst formation.

Materials and methods

Isolation of follicles for culture

The use of mice for our study was approved by the Institutional Animal Care and Use Committee under IRB Protocol 9803-494A. B6D2F1 mice were used for this study. All female mice were 14 days old, and all males were between the ages of 6 and 10 weeks. Early preantral follicles were isolated as published previously [1, 2]. Female mice were sacrificed by cervical dislocation and ovaries were removed and stored in Hank's balanced salt solution with 10% fetal calf serum (FCS). Early preantral follicles were mechanically dissected from the ovaries using 28 in. × 0.5 in. 18G syringe needles (Becton Dickinson and Co., Franklin Lakes, NJ). Follicles (100–120 μM in diameter) containing layers of membrane-enclosed granulosa cells with a centrally located, healthy, visible oocyte and attached with thecal cells were selected for study. The isolated preantral follicles were then cultured in microdroplets (20 μL) in a culture dish (Falcon 3002, 60 mm × 15 mm, 1 follicle per droplet, 30 droplets per dish) with medium, covered with washed mineral oil and incubated at 37°C in 5% CO_2 for 10 days. These follicles were first cultured with Opti-MEM medium (Gibco, Grand Island, NY) plus recombinant LH (100 mIU/mL) and recombinant FSH (100 mIU/mL) plus 5% FCS for 2 days, then changed to Opti-MEM medium, plus recombinant LH (10 mIU/mL) and recombinant FSH (100 mIU/mL) plus 5% FCS for 8 days. Media was changed by refreshing half (10 μL) of the medium every day. Media were collected for detection of follicular E_2 secretion by radioimmunoassay. Morphological changes of the cultured follicles were evaluated and videotaped under an inverted microscope (Diaphot-TMD, Morrell, NY).

Ovulation in-vitro and classification of the released oocytes

During 10 days of IVC/IVM, the preantral follicles gradually transformed into fully developed antral follicles. On day 10, administration of 1.5 IU/mL of recombinant hCG, plus 5 μg/mL of human epidermal growth factor (EGF) would trigger ovulation in-vitro to release cumulus-oocyte complexes from the antral follicles. The released oocytes were classified as GV when the germinal vesicle (GV) was present, as metaphase I (MI) when GV was broken down, as metaphase II (MII) when the first polar body was extruded, and as degenerated when oocytes were dark, granulated, or fragmented.

In-vitro fertilization and early embryo development

The released MII oocytes were fertilized in Ham's F10 medium (Gibco) containing 1×10^6/mL of spermatozoa. Three hours after fertilization, oocytes were washed and cultured singly in 20 μL droplets with M16 (Sigma, St. Louis, MO) medium plus 10% FCS for 5 days. After fertilization, early development into 1-cell, 2-cell, 4-cell, 8-cells, morula and blastocyst stages were carefully monitored for analysis.

Assessment of individual follicular E_2 secretion

Media collected from all the droplets were used to measure E_2 concentration by radioimmunoassay using a commercially available direct E_2 assay kit (Pantex, CA). The sensitivity of the assay was 10 pg/mL, and the intra-assay and inter-assay coefficients were 7 and 10%, respectively. Samples were measured after an appropriate dilution with kit's zero standard, which was steroid free.

Statistics

Category variables were assessed by calculating x^2 with Yates's correction, or Fisher's exact test in the case of small cell frequencies. $P < 0.05$ was considered statistically significant.

Results

Follicular development during maturation in-vitro

In our culture condition, isolated preantral follicles (Fig. 1a) underwent stepwise morphological changes during in-vitro maturation. These changes include: the attachment and proliferation of thecal cells from day 2, proliferation and outgrowth of granulosa cells from day 3 (Fig. 1b), and granulosa cell differentiation and antral formation from day 6 (Fig. 1c). Ovulation (Fig. 1d) and mucification of GV stage oocytes occurred upon the stimulation of hCG/EGF on day 10. After ovulation, GV stage oocytes resumed meiosis to complete nuclear maturation by GV breakdown (classified as GVBD), followed by the extrusion of the first polar body (classified as MII) (Fig. 1e). Some MII oocytes can be fertilized, cleaved, and advanced to the 2-cell, 4-cell, and conceivably to blastocyst-staged embryos (Fig. 1f).

Outcome of follicles matured in-vitro

In this study, seventy-eight follicles were isolated from 4 female mice and matured in-vitro. After in-vitro maturation and ovulation, 78 oocytes were generated. 5 were degenerated, 22 were at the GV stage, 15 at the GVBD stage and 36 at the MII stage. Of 36 MII-staged oocytes, 21 were fertilized and 13 developed into blastocyst-staged embryos. The fertilization and blastocyst formation rates were 58.3 and 36.3%, respectively (Table 1).

Follicular E_2 secretion concentration and patterns

In general, cultured follicles initiated secretion of Estradiol beginning on day 4. This secretion increased progressively as the follicles grew to more advanced stages. When it approached final maturation (usually around the day of hCG administration), variations in secretion patterns were observed. Secretions were classified in relation to the day of hCG as the following: Pattern A: E_2 level was slightly decreased after hCG administration; Pattern B: E_2 level reached plateau

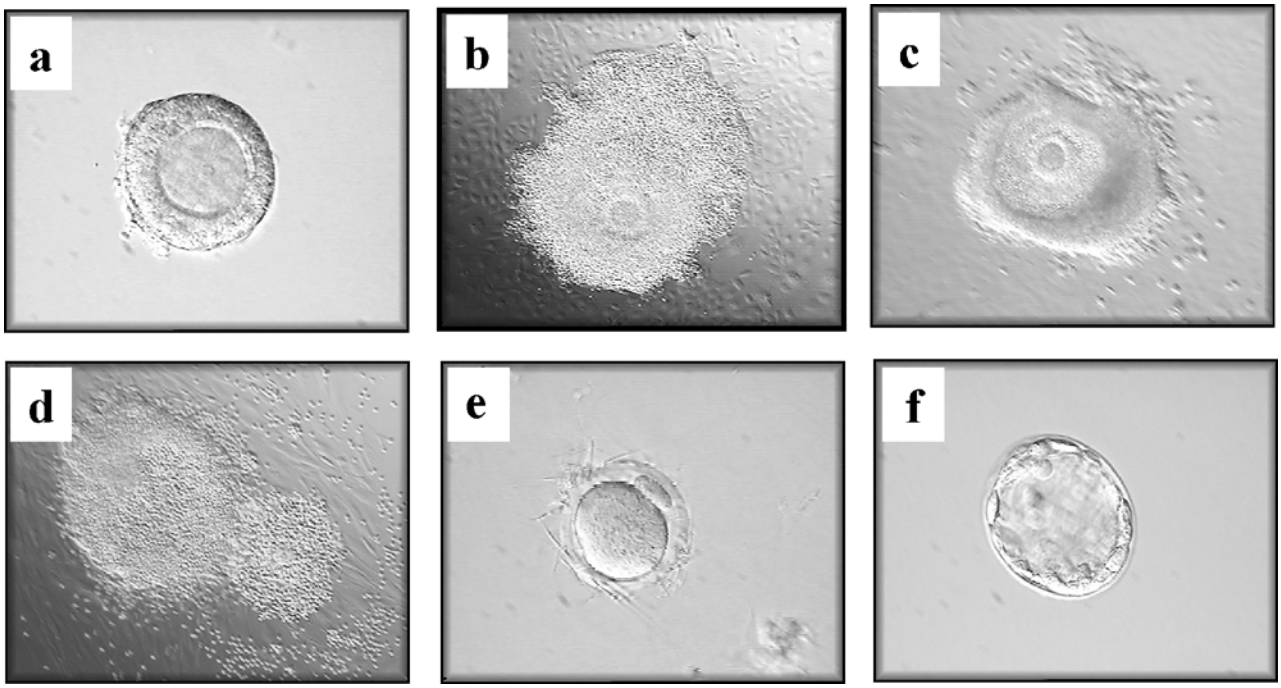

Fig. 1 In-vitro mouse follicular development: The isolated preantral follicles (a) matured in-vitro with recombinant gonadotropins underwent stepwise morphological changes. These changes include the attachment and proliferation of thecal cell from day 2, proliferation and outgrowth of granulosa cells from day 2 (b), granulosa cell differentiation and antral formation from day 6 (c), ovulation and mucification of GV stage oocytes upon the stimulation of hCG/EGF on day 10. After ovulation, the GV stage oocytes resumed meiosis to complete nuclear matuartion by GV breakdown (classified as GVBD), followed by the extrusion of the first polar body (classified as MII) (e). These MII oocytes can be fertilized, cleaved, and advanced to the 2-cell, 4-cell and conceivably to the blastocyst stage embryo (f)

Table 1 Follicles matured in vitro and types of oocytes generated

	#	A (%)	E (%)	F (%)
A. Follicle	78			
B. Degenerated	5	6.4		
C. GV	22	28.2		
D. GVBD	15	19.3		
E. MII	36	46.2		
F. Fertilized	22	26.9	58.3	
G. Blastocyst	12	15.4	33.3	54.6

before hCG administration and decreased after hCG administration; Pattern C: E_2 level decreased before hCG administration and increased after hCG administration; Pattern D: E_2 level decreased before hCG administration and continued to decrease after hCG administration; and pattern E: E_2 level sharply decreased (>30%) after hCG administration (Fig. 2).

There was a good correlation between somatic cell steroidogenesis and maturity of oocytes. Most of the degenerated oocytes exhibited E_2 production (on day of hCG) between 0 and 40 ng/mL; most of the GV staged-oocytes exhibited E_2 production between 30 and 50 ng/mL; most of the GVBD-staged oocytes exhibited E_2 production between 40 and 60 ng/mL; and finally, most of the MII-staged oocytes exhibited E_2 production between 50 and 70 ng/mL (Table 2). The mean E_2 secretion concentrations of these degenerated, GV-staged, GVBD-staged, and MII-staged oocytes were 42.8, 47.2, 53.7 and 68.7 ng/mL respectively (Table 3). The majority of the follicles matured in-vitro exhibited E_2 secretions with Pattern A. In this study, thirteen of the GV oocytes, 12 of the GVBD oocytes and 32 of the MII oocytes were associated with an A secretion pattern (Table 3, Fig. 2). Only one GVBD and one MII oocyte were associated with pattern B secretion. Two degenerated and two GV oocytes were associated with a pattern C secretion. One degenerated and one GV oocyte were associated with a D secretion pattern. Two degenerated, 6 GV, 2 GVBD and 3 MII oocytes were associated with an E secretion pattern (Table 3, Fig. 2). Interestingly, 4 MII oocytes not associated with an A secretion pattern (3 were associated with an E

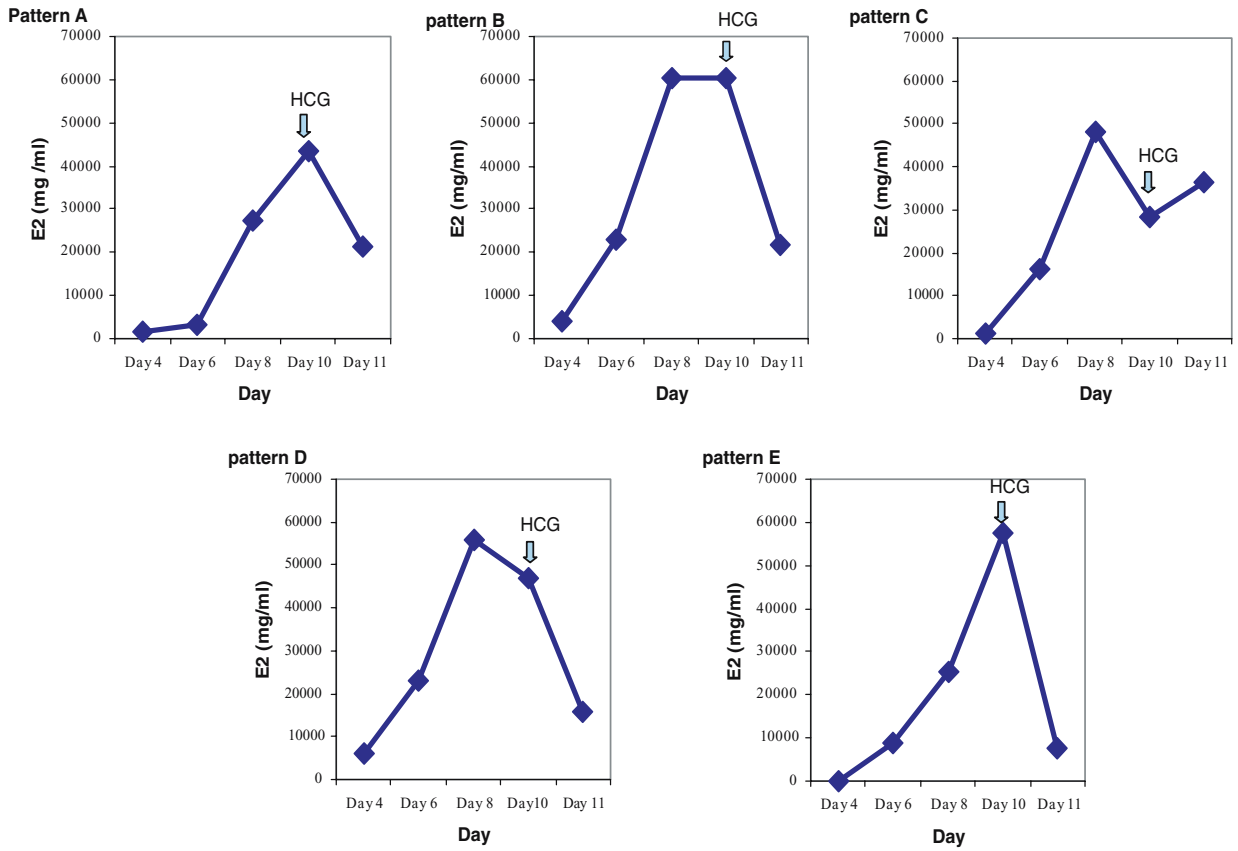

Fig. 2 Follicular E_2 secretion patterns: Follicular E_2 secretion patterns were classified in relation to the day of hCG as: Pattern A: E_2 level was slightly decreased after hCG administration. Pattern B: E_2 level reached plateau before hCG administration and decreased after hCG administration. Pattern C: E_2 level decreased before hCG administration and increased after hCG administration. Pattern D: E_2 2 level decreased before hCG administration and continued to decrease after hCG administration. Pattern E: E_2 level decreased sharply (>30%) after hCG administration

Table 2 Correlation between follicular steroidogenesis and types of oocytes generated

E_2 on day of hCG (ng/mL)	Types of oocytes			
	Deg. ($n=5$)	GV ($n=22$)	GVBD ($n=15$)	MII ($n=36$)
≤ 30.0	2	3	0	0
30.1–40.0	1	7	1	2
40.1–50.0	5	4	4	
50.1–60.0	1	1	8	10
60.1–70.0	2	1	16	
70.1–80.0	2	4		
≥ 80.1	1	2	1	
	5	22	15	36

secretion pattern and one was associated with a B secretion pattern) were either not fertilized, or fertilized but became degenerated later, an indication of poor quality (Table 3, Fig. 2).

Predictability of follicular E_2 production and pattern form blastocyst formation

The potential of embryo development varied among 32 MII oocytes associated with an A secretion pattern. After fertilization, 11 were not fertilized, 4 were fertilized but degenerated later, 5 were fertilized but did not develop beyond the morula stage, and only 12 were fertilized and developed to the blastocyst stage.

Eighteen of these 32 MII oocytes were ovulated when their follicular E_2 secretion was at the range of 60–80 ng/mL. Of them, 13 were fertilized and 10 developed into blastocysts after culture. Therefore, these MII oocytes exhibited a fertilization rate and blastocyst formation rate of 72.2 and 55.6%, respectively (Table 4).

On the other hand, 14 of these 32 MII oocytes were ovulated when their follicular E_2 production was either ≤ 60 ng/mL or ≥ 80 ng/mL (i.e., Group II). Of them, 8 were fertilized and 2 developed into blastocysts. Thus, their fertilization and blastocyst formation rates were 57.1 and 14.3%, respectively (Table 4). The blastocyst formation rate was significantly different in Group I and Group II ($p < 0.0167$) (Table 4). Our data strongly suggests that somatic cell E_2 secretions and patterns can be a predictor for the quality of their embraced oocytes. Using a combination of follicular E_2 production of 60–80 ng/mL at ovulation and an A secretion pattern, the predictability for blastocyst formation was 83.3%, with a sensitivity of 55.6% and specificity of 14.3% (Table 4).

Repeat experiments

In our second study group, 17 female mice were sacrificed and 101 preantral follicles were isolated. After in-vitro maturation and ovulation, 100 oocytes were generated. Fifty-five of them were at the MII-stage and 25 were associated with an A secretion pattern with a follicular E_2 secretion ranging from 60–80 ng/mL. Of these 25 MII oocytes, 19 were fertilized and 15 developed to blastocysts. These results further confirmed that a combination of follicular E_2 secretion pattern and level are good markers for generating competent oocytes after in-vitro maturation of mouse follicles. Results of both experiments were summarized in Table 5.

Discussion

In the past, we have successfully established an in-vitro culture system that allows for maturation of mouse preantral follicles with recombinant gonadotropins and growth factors [1, 2]. Only those follicles >100 μM in diameter size, attached with theca cells, and containing a viable oocyte that was surrounded by several layers of granulosa cells were selected for study. The integrity of theca cell-granulosa cell-oocyte unit facilitates endocrine and paracrine responses and transportation, also allowing for proper cell to cell interactions in order to trigger the physiological and biological

Table 3 Patterns of E_2 secretion play a role in oocyte maturation

Type of oocyte	#	E_2 (ng/mL) $X \pm$ SD	Secretion patterns				
			A (%)	B (%)	C (%)	D (%)	E (%)
Degenerated	5	42.8 ± 35.7	0 (0)	0 (0)	2 (40)	1 (20)	2 (40)
GV	22	47.2 ± 20.0	13 (59.1)	0 (0)	2 (91)	1 (4.6)	6 (27.3)
GVBD	15	53.7 ± 11.3	12 (80)	1 (6.7)	0 (0)	0 (0)	2 (13.3)
MII	36	68.7 ± 11.5	32 (88.9)	1 (2.8)	0 (0)	0 (0)	3 (8.3)

Table 4 Predictability of follicular E_2 production and pattern for blastocyst formation

	Group I[a]	Group II[b]	*P* value
A. MII	18	14	
B. Fertilized (% of MII)	13 (72.2)	8 (57.1)	
C. Blastocyst (% of MII)	10 (55.6)	2 (14.3)	0.0167
D. Failed to form blastocyst (% of MII)	8 (61.5)	12 (80.0)	

Note. Sensitivity for blastocyst formation: 10 (10 + 8) = 55.6%, Specificity for blastocyst formation: 2 (2 + 12) = 14.3%, Predictability for blastocyst formation: 10 (10 + 2) = 83.3%

[a]Group I includes MII oocytes derived from follicles with follicular E_2 production of 60–80 ng/mL

[b]Group II includes all other studied MII oocytes

changes. During IVC/IVM, cultured follicles underwent dramatic morphological changes and oocyte maturation in a coordinated manner (Fig. 1). Concurrently, granulosa and thecal cells under the stimulation of exogenous FSH and LH produced E_2 progressively (Fig. 2). Although not perfect, our follicular IVC/IVM system allows follicular cell types to remain in contact, antral-like cavities to be formed, responsiveness to gonadoptropin stimulation to be preserved, and more importantly, competent oocytes were able to be generated (Table 1).

In this study, 78 follicles with similar morphological and developmental stages were cultured in the same culture conditions, yet they developed into follicles producing a wide range of Estradiol concentration (ranges from 8 to 95 ng/mL) and were associated with oocytes with varying stages of maturity (i.e. deg, GV, GVBD, and MII) (Table 1). This data suggests that these follicles were indeed different individually, possibly with different amounts of gonadotropin receptors, leading to their dramatic differences in responses. The ones that responded well, tend to produce high Estradiol concentration and be associated with mature oocytes. The mean follicular E_2 production increased in stepwise fashion as the associated oocytes advanced from degenerated to GV, GVBD and MII stages (Tables 2 and 3). Estradiol may promote the development of follicles by its stimulation of proliferation and differentiation of somatic cells and perhaps by its anti-apoptotic effects as well [19]. A drop in follicular Estradiol could happen during follicular growth (i.e. C and D secretion pattern) or at the end of growth (i.e. E secretion pattern). It is not known what could cause the drop of follicular E_2 production. It might be attributed to atresia, poor proliferation or differentiation of granulosa cells. A drop in follicular Estradiol production during the growing phase of follicles (i.e., C and D secretion patterns) were often associated with degenerated or immature oocytes (Table 3). A drop of follicular E_2 production at ovulation (i.e., E secretion pattern) might be associated with all types of oocytes (i.e., degenerated, GV, GVBD and MII oocytes) indicating that atresia could occur virtually at any stage of oocyte maturation, resulting in poor quality oocytes. Of 3 MII oocytes associated with E secretion pattern, 2 were not fertilized, and one was fertilized but degenerated after fertilization. Most (32/36) of MII oocytes, most (21/22) of fertilized MII oocytes and all blastocysts (12/12) were associated with an A secretion pattern. Obviously active follicular E_2 production (i.e, A secretion pattern) prior to ovulation was essential for the development of follicles.

These data confirms the important role of somatic cell steroidogenesis during follicular development. During maturation, as the preantral follicles advanced to antral follicles, somatic cells undergo differentiation and becomes progressively more responsive to gonadotropin stimulation. Thus, the development of somatic cell steroidogenic function and the maintenance of their steroidogenesis activity prior to ovulation can be used as a functional marker of normality of follicular development [20, 21]. With normal development of follicles, both nuclear and cytoplasmic maturation were acquired sequentially and reached their full capacity eventually [15, 22]. However, active follicular E_2 secretion prior to ovulation alone did not ensure developmental capacity of mature oocytes.

Of 32 MII oocytes associated with an A secretion pattern, 11 were not fertilized, 4 were fertilized but degenerated later, 5 were fertilized but did not develop beyond the morula stage, and only 12 were fertilized and developed to the blastocyst stage. Therefore, these MII oocytes had the potential to complete meiotic maturation, yet they were indeed different in their developmental capacity. The developmental capacity of mature oocytes depends on 1) Oocyte capacitation which is

Table 5 Summary results

	Experiment 1	Experiment 2	Total
A. # of female mice used	4	17	21
B. # follicles isolated	78	101	179
C. # oocytes generated	78	100	178
D. # MII	36	55	91
E. # MII with A secretion pattern and secretion levels of 60–80 ng/mL	18	25	43
F. # oocytes fertilized (%)	13 (72.2)[a]	19 (76)[a]	32 (74.4)[a]
G. # blastocysts (%)	19 (76.0)[b]	15 (78.9)[b]	25 (78.1)[b]

[a]"()" expressed as %E [b]"()" expressed as %F

the preparation of oocytes during follicular development for supporting early embryo development and 2) the morphological and biochemical modification of the oocyte during maturation, which were triggered by the LH surge or by the administration of hCG [15]. In normal folliculogenesis, both nuclear maturation and cytoplasmic competency were acquired incrementally until their full capacity. During this period of time, the key nuclear maturation proteins, cell cycle proteins and maternal mRNA were sequentially synthesized and accumulated to ensure the capacity to develop beyond the activation of embryonic genome and pass through the transition from morula to blastocyst during preimplantation embryo development [23].

In IVC/IVM, hCG was administered to induce final nuclear and cytoplasmic maturation. The steroid milieu of oocytes at the time of hCG administration is critical. To obtain competent oocytes, hCG should be administered at the time oocytes acquire its cytoplasmic competency. The cytoplasmic competency might be optimal when follicular E_2 production was within the range of 60–80 ng/mL. Most of the blastocysts (10/12) resulted when hCG was administered within the range and only a few blastocysts (2/12) were obtained when hCG was administered out of the range (Table 4). The blastocyst formation rate of follicles producing follicular E_2 of 60–80 ng/mL was significantly higher than that of the follicles producing follicular $E_2 < 60$ ng/mL or >80 ng/mL (55.6% vs. 14.3%, $p < 0.0167$) (Table 5). If follicular E_2 production of 60–80 ng/mL was indicative of optimal cytoplasmic maturation, the cytoplasm might then be immature with E_2 production of <60 ng/mL and was post mature with E_2 production of >80 ng/mL. Cytoplasmic competency was significantly reduced when oocytes were either immature or postmature. Therefore, cytoplasmic maturation was the key factor in supporting further development of the fertilized oocytes to the blastocyst stage. A competent oocyte, requires a well synchronization between cytoplasmic and nuclear maturation at the time of hCG administration [24].

Our data strongly suggests that both active follicular E_2 production prior to ovulation and optimal steroid milieu at ovulation were two equally important factors for competent oocyte generation and a combination of somatic cell steroid concentration and pattern can be a good prognosticator for a high yield of competent oocytes after in-vitro follicular stimulation with recombinant gonadotropins. Their predictability for blastocyst formation was as high as 83.3% (Table 4).

In this study, hCG was designed to be administered on D10. If hCG could be administered one day early (i.e., D9) for the follicles with high E_2 production, or one day later (i.e., day 11) for the follicles with low E_2 production, the efficiency of our IVC/IVM might have been improved further.

Though the optimal follicular E_2 secretion range for cytoplasmic maturation had been identified by this preliminary study, it needs to be redefined or confirmed with a large number of samples. Whether it could be affected by different stimulation regimes during in-vitro maturation requires further study as well.

In conclusion, mechanically isolated early preantral mouse follicles can be stimulated and matured in-vitro with gonadotropins. During in-vitro culture/in-vitro maturation (IVC/IVM), oogenesis occurs concomitantly with folliculogenesis in a coordinated manner. Upon the stimulation of administered gonadotropins, somatic cells (i.e. both thecal and granulosa cells) respond to synthesize Estradiol. Active production of Estradiol (i.e., secretion A pattern) before ovulation is essential to maintain the viability of the cultured follicles. To obtain competent oocytes, an optimal steroid milieu at ovulation is required to ensure well synchronization between nuclear and cytoplasmic maturation. Therefore, a combination of active somatic cell steroid pattern prior to ovulation (A secretion pattern) and concentration (60–80 ng/mL) at ovulation can be used as an indicator for competent oocytes. With this indicator, the predictability for blastocyst formation was as high as 83.3%. Using somatic cell steroidogenesis as guidelines, our mouse follicular IVC/IVM may be further refined and high yield of competent oocytes may be obtained.

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